BIOCHEMICAL STUDIES OF LIPOXIDASE IN SEEDS AND SEEDLINGS OF SUNFLOWER, *HELIANTHUS ANNUUS* (L.)

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April, 1958

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BIOCHEMICAL STUDIES OF LIPOXIDASE IN SEEDS AND SEEDLINGS OF SUNFLOWER, HELIANTHUS ANNUUS (L.)

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Biochemical Studies of Lipoxidase in Seeds and Seedlings of Sunflower, Helianthus annuus (L.)

(Abstract)

Lipoxidase activity was obtained in enzyme preparations from sunflower seed and seedlings. A partly purified preparation from seedlings was used for enzyme kinetic studies. The pH optimum was 6.8, and 100% oxygen was required for maximum activity. The Michaelis constant, with potassium linoleate as substrate, was 1.64×10^{-3} M. The reaction products were conjugated dienes. Enzyme activity was not affected by various metal and sulfhydryl inhibitors, nor by α-tocopherol, but catechol, α-naphthol, ethanol and potassium oleate were inhibitory. Oil from flax, rape, and sunflower seeds reduced total oxidation of linoleate by the enzyme. Copper sulfate increased the rate and total oxidation of the linoleate-lipoxidase system, but iron, manganese, magnesium and calcium were without effect. Lipoxidase activity was associated with mitochondrial (15,000 x g), intermediate (25,000 x g), and microsomal (100,000 x g) fractions, as well as with the soluble cytoplasmic proteins. Lipoxidase activity in seedlings increased during initial stages of germination, then decreased. The most rapid depletion of total fat in the seedlings coincided with maximum lipoxidase activity.

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INTRODUCTION

Lipoxidase is the only enzyme known to catalyze specifically the oxidation of linoleic and linolenic acids in plants. This enzyme was discovered first in soybean, but has also been found in other plants. It appears that lipoxidase is particularly abundant in seeds and seedlings, but little information is available on the role of this enzyme in the metabolism of lipids in plants. Many oil seeds, including sunflower, were claimed to be lipoxidase negative (17, 50). However, in a preliminary survey on lipoxidases from seeds of various plant species, an active enzyme was obtained from sunflower seeds. Since sunflower lipoxidase had not been studied previously, it was selected as the source of lipoxidase for these investigations.

The work reported in this thesis deals with the following aspects of sunflower lipoxidase:

1. The activity of lipoxidase from seeds.
2. The relative lipoxidase activity in seedlings during the early stages of germination.
3. The distribution and association of the enzyme in cell fractions.
4. The characterization of a partly purified preparation of lipoxidase from sunflower seedlings.

LITERATURE REVIEW

I. Essential Fatty Acid Oxidation

Lipoxidase is the only enzyme which has been shown to catalyze the oxidation of polyunsaturated fatty acids in plants. It was discovered first in soybean (4), but has been shown to be present in many other plants (16, 50). The primary product of the oxidation is a conjugated diene hydroperoxide with a maximum absorption in the ultraviolet spectrum at 234 mμ (45, 49).

The specific substrates for lipoxidase are linoleic, linolenic and arachidonic acids (47, 50). These acids are polyunsaturated and comprise the essential fatty acids, the only compounds which will restore growth and cure skin defects in animals lacking fat in their diet (61). Arachidonic acid is the most effective of the essential fatty acids. It is found only in animal fats, and is derived from linoleic acid (47). Synthesis of linoleic and linolenic acids have been shown to occur in plants (23), but the function of these acids in plants is not known. They resemble vitamins in their specific deficiency symptoms, but are generally required in larger amounts (47). Tocopherol (vitamin E) is a natural antioxidant and is associated with the regulation of oxidation of essential fatty acids, but the metabolic

significance of this association is not clear (47).

The essential fatty acids, as well as oleic acid, autoxidize readily in air. Autoxidation is a free radical chain reaction, and among the products are the characteristic diene hydroperoxides of the lipoxidase-catalyzed oxidation of unsaturated fats (4). Autoxidation is one of the causes of rancidity in fats and oils, the off-odors and tastes of rancid fat being due to the presence of aldehydes and ketones formed as secondary products of autoxidation (4).

Certain specific compounds in biological systems also will catalyze the oxidation of essential fatty acids. Tappel made a comparative study of heme-containing compounds (54, 56). He found that linoleate oxidation was catalyzed by cytochrome C, hematin, hemoglobin, and catalase. Catalase was the least effective catalyst. Collier and McRae (9) also observed linoleate oxidation catalyzed by cytochrome C. The primary products of hematin-catalyzed oxidation also were conjugated dienes (56). Tappel (56) was able to show that degradation of the hydroperoxide and simultaneous destruction of hematin occurred in the dark in an oxygen-free atmosphere. The presence of light and increasing temperature promoted the destructive processes. During the reaction ketonic products accumulated.

The oxidation of unsaturated fats and fatty acids also may be catalyzed by other compounds and factors. Copper

proteins greatly accelerate the autoxidation and diene formation of methyl linoleate (57). Copper oleate, as well as ultra-violet light and visible light, are effective catalysts (35). Chlorophyll catalyzes methyl-linoleate oxidation in the presence of light. The products include non-conjugated hydroxyl compounds (35). In addition, conjugated dienes can be obtained by heating the unsaturated acids in potassium hydroxide (43).

II. Lipoxidase

(a) Occurrence

In 1932, Andre and Hou (cit. 29) applied the name lipoxidase to a fat-oxidizing enzyme system in soybean. Since that time the enzyme has been found to be present in many other plants. Siddiqi and Tappel (48, 49, 50) have provided evidence of an active lipoxidase in defatted meals from pea, soybean, urd bean, mung bean, and peanut, as well as in leaf juice from alfalfa. Irvine and Anderson (32) showed unequivocally the presence of lipoxidase in wheat. In a study of lipoxidase in cereal crops, Franke and Frehse (16) obtained evidence of this enzyme in barley, rye, wheat, oats and corn. The enzyme has been noted in flax seed, sunflower and rape seedlings (17), and in potato (21). Sunflower and rape seed did not possess an active lipoxidase (17, 53).

Many attempts have been made to show the presence of lipoxidase in animal tissues. Destruction of β-carotene and vitamin A, as well as rancidity, are associated with unsaturated fat oxidation (46, 62). Consequently, these phenomena were taken as evidence of the presence of active lipoxidase. Thus, Brocklesby and Rogers (8) observed a very active vitamin-A-destroying entity in salmon liver. Reiser (46) reports on a peroxidizing, β-carotene destroying heat labile substance in bacon. Collier and McRae (9) attributed the catalytic activity of an erythrocyte hemolyzate to the hemoglobins. They found no evidence of lipoxidase activity. The results by Boyd and Adams (7) strongly suggest that catalyzed oxidation of unsaturated fats in animal tissues is effected by heme compounds and that lipoxidase is absent.

The information on lipoxidase in bacteria and fungi is very limited. Fucuba (21) observed linoleate oxidation in Aspergillus and Rhizopus, but not in bacteria. Franke (14), in his extensive review, cites data by Franke and Schillinger which indicate that gram-positive bacteria oxidize saturated as well as unsaturated long-chain fatty acids, but the products were not analyzed. There is thus very little evidence for lipoxidase in microorganisms.

(b) Practical Importance

Interest in lipoxidase arose from its effect on natural pigments. Vitamin A was found to be destroyed in

alfalfa (41), and pigments in flour were bleached, especially in the presence of a legume seed extract (29). It was recognized that an enzymic factor was responsible; moreover, the necessity for simultaneous unsaturated fat oxidation was established (53).

Lipoxidase may be at least partly responsible for oxidative rancidity during flour storage and for the destruction of yellow pigments (31, 40).

Use has been made of lipoxidase as a bleaching agent in bread-making. Haas and Bohn (25) patented this method of bleaching bread dough. Chemical bleaching agents are frequently used, but bleaching by lipoxidase has some advantage. Thus it was observed by Fucuba (21) that lipoxidase destroys primarily vitamin A, whereas Novadolox also destroys some of the B-vitamins.

Bleaching of pigments is desirable in bread dough, but not in macaroni dough. Carotenoid pigments contribute significantly to the yellow color of macaroni. Irvine and Winkler (31) made a comprehensive study of the enzymatic destruction of pigments during dough-mixing. Irvine has concluded that lipoxidase is responsible for the low correlation between pigment content of semolina and the color of macaroni (30).

Lipoxidase has also been held responsible for vitamin A destruction in fish oil (8); but the possibility

cannot be excluded that a heme compound catalyzes the reaction (54).

(c) Physiology of Plant Lipoxidases

Experimental evidence seems to indicate that lipoxidase is very prevalent in seed, and particularly in seedlings during the first days of germination. Fritz and Beevers (19) observed maximum lipoxidase activity in corn after 2 - 3 days' germination. Holman (28) found that lipoxidase activity in soybean decreased rapidly after the third day; furthermore, in barley seedlings, no lipoxidase activity could be observed after 12 - 13 days (16).

Franke and Frehse (16) made a study of specific lipoxidase activity in barley grains during maturation. Their results showed a tenfold increase in active lipoxidase from the milk-ripe stage to the fully ripe stage.

Sissakian (51) has reported a variation in lipoxidase activity in carrot chromoplasts during storage. Active lipoxidase decreased in a variety of poor keeping quality, whereas a good storage variety had no lipoxidase in the fall but increasing amounts in early spring. Sissakian (51) also presented evidence for an oxidase in isolated chloroplasts which oxidized the essential fatty acids as well as palmitic and oleic acids. The oxidation did not require exogenous diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP). That

lipoxidase is present in green portions of plants was also shown by Siddiqi and Tappel (48). Potato tubers contain an enzyme which can catalyze dye oxidation and consequently mediate reduction of the dye (20). This enzyme was shown to be lipoxidase. In general, it appears that lipoxidase is comparatively less abundant in roots, leaves and shoots than in seeds and young seedlings (14).

(d) Metabolic Function

Holman and Bergstrom (29), in their comprehensive review, stated: "The role of lipoxidase in metabolism is unknown." More information has been acquired since that time, but the function of lipoxidase in cellular metabolism still is a matter of speculation.

The enzyme has nearly always been associated with the supernatant fraction of cell-free homogenates (19, 39). Fritz and Beevers (19) were able to show that lipoxidase was associated with the supernatant soluble portion, but that a natural substrate was present in a mitochondrial fraction. Pea mitochondria failed to catalyze the oxidation of methyl linoleate (39). However, Goodwin and Waygood (24) identified lipoxidase activity in barley mitochondria. Consequently, the enzyme may not be confined to a specific cell fraction.

Mapson and Moustafa (39) have found evidence of a lipoxidase-linoleate-catalyzed oxidation of glutathione.

They also observed that long-chain aliphatic alcohols had a stimulating effect on lipoxidase activity of a natural substrate. After six days of germination, this activating effect was eliminated. It indicated that during germination changes occurred which resembled those induced by the action of alcohol. It may have been a "loosening" of lipoxidase, as proposed by Sissakian (51) for other enzymes, during developmental changes after the resting stage.

The results reported by Goodwin and Waygood (24) indicate that during germination there is a gradual increase in lecithinase C activity, and a concomitant increase in active lipoxidase. Lecithinase C breaks down lecithin to phosphorylcholine and diglyceride. The latter compound may then act as a substrate for lipoxidase. Franke and Frehse (17) were unable to show active lipoxidase in rape and poppy seeds, whereas rape seedlings contained active lipoxidase. However, a heat-stable petroleum ether insoluble compound was present in rape seeds, which partly curtailed the activity of barley lipoxidase.

Although the specific metabolic function of lipoxidase has not been elucidated, there have been several suggestions as to the possible significance of the enzyme. Holman (28) proposed that lipoxidase acted to initiate the oxidation of unsaturated fats by catalyzing the formation of free radicals. Fritz and Beevers (20) concluded that it may act as a terminal oxidase. In their system, lipoxidase was shown

to oxidize 2,3',6-trichlorophenolindophenol and mediate a subsequent irreversible reduction. These authors point out that the dye may have served as an artificial carrier, and that some other compound was oxidized through the lipoxidase system and the carrier. This hypothesis agrees with results obtained by Mapson and Moustafa (39), indicating that oxidation of reduced glutathione by the linoleate-lipoxidase system had taken place. However, Fritz and Beevers (19), in their postulation, implied that triphosphopyridine nucleotide (TPN) was oxidized by oxygen through the lipoxidase system; but Mapson (38) found that TPN appears to be destroyed during enzymatic reduction involving TPN, if both oxygen and lipoxidase are present. Tappel et al. (59) found that the antioxidant (nordihydroguaiaretic acid) (NDGA) was oxidized with no apparent net oxidation of linoleate, but the lipoxidase-linoleate system was required. NDGA was later shown to act as a competitive inhibitor for lipoxidase (50). Thus the basic function of lipoxidase appears to be the extraction of an electron or a hydrogen from the methylene group of the essential fatty acids, but the metabolic significance of this process is unknown.

There have been some reports suggesting a deleterious effect on other enzyme systems by lipoxidase and fatty acid peroxides. Goodwin and Waygood (24) found a decrease in succinoxidase activity, with an increase in lecithinase C and lipoxidase activity. The destruction of succinoxidase was attributed to the destruction of the functional integrity of

the enzyme complex in mitochondria as a result of hydrolysis by lecithinase C and subsequent peroxidation of the unsaturated diglyceride by lipoxidase,

Bernheim et al. (5) reported that rat liver succinoxidase, cytochrome a and choline oxidase are inactivated by methyl linolenate previously exposed to ultra-violet light. This suggests that peroxidized fatty acids have an adverse effect on some enzyme systems.

Lipodehydrogenase

Franke and Frehse (17) have reported on the presence of lipodehydrogenases in plants. They observed that seeds high in lipoxidase activity also contained an enzymic factor which could catalyze the reduction of a dye under anaerobic conditions in the presence of unsaturated fatty acids, and to a limited extent in the presence of the saturated long-chain fatty acids. Seeds which lacked active lipoxidase did not reduce the dye in the presence of linoleate and linolenate, but exhibited some reductive activity in the presence of oleate and particularly with palmitate and stearate as substrates. After the germination process had begun, the essential fatty acids also were effective as substrates for the second group.

In a detailed study of barley lipodehydrogenase, Franke and Frehse (17) found that the enzyme was associated with the soluble fraction of the homogenate. The enzyme is soluble in water, retains its activity after dialysis, and can

utilize oleate, linoleate, linolenate and the C_{10}-C_{16} saturated fatty acids during reduction of the dye. There was no clear indication that DPN, ATP or coenzyme A were involved in the reaction.

The decline of lipodehydrogenase activity in barley during germination tends to be more rapid than that of lipoxidase activity (17). This is particularly evident during the first three days of germination. In spite of the similarity in many respects between lipoxidase and lipodehydrogenase, the authors claim that two specific enzymes are involved. They observed that destruction of lipoxidase did not hinder the dye reduction process. Moreover, they were able to show that lipoxidase-oxidized linoleate was dehydrogenated at a higher rate than fresh linoleate. This activation was at a maximum when 10% of the acid was oxidized. On the basis of their results with lipoxidase and lipodehydrogenase Franke and Frehse (17) proposed that lipoxidase may function to initiate through peroxide formation the degradation of the higher unsaturated fatty acids by β-oxidation.

(e) Methods of Measurement

Several methods have been employed to measure the activity of lipoxidase. The one most commonly used is the Warburg manometric technique (60). It measures the amount of oxygen utilized by lipoxidase during the oxidation of the substrate. This method has been used by Franke and Frehse (16),

Siddiqi and Tappel (49), and others (32, 36, 39).

The initial products of linoleate-lipoxidase oxidation are conjugated dienes. These compounds absorb strongly in the ultra-violet spectrum (43). Hence the increase in diene formation measured at 230 - 234 mμ has also been used to study the activity of lipoxidase (52, 59).

Destruction of pigments also has provided a means of measuring lipoxidase activity. β-carotene (29), bixin (37) and crocin (21) have been used with success. It was observed that the amounts of conjugated diene formed and carotene destroyed were proportional to enzyme concentration and to time. The destruction of the pigments is followed spectrophotometrically. This method, however, is effective only within rather narrow limits of carotene concentrations.

Infra-red spectroscopy has been used to identify the products (35). Different chemical groupings or configurations of molecules vary in their absorption spectra in the infra-red portion of the spectrum (44). The specific absorption spectrum depends on the vibrational levels of the groups. The method has been employed to establish the changes in configuration of the essential fatty acids during oxidation (34, 35, 45).

Aliphatic compounds such as occur in the products of fatty acid oxidation can be separated on the basis of differences in chain length, type of functional group, kind and

degree of unsaturation, as well as branching or polar groups along the chain. This separation has been utilized by Khan et al. (35), who employed displacement chromatography in conjunction with infra-red and ultra-violet spectral analyses to identify oxidation products.

(f) Biochemistry and Kinetics

Soybean lipoxidase has been purified and obtained in crystalline form (27). It is a typical globulin, very soluble in dilute salt solutions. Its molecular weight is 102,000, and its isoelectric point is at pH 5.4. The enzyme is unique as an oxidase, because it has no prosthetic group, nor does it require any coenzyme (27). Lipoxidases from other plant sources have been studied in detail, but have never been purified to the crystalline stage (16, 32, 49).

Influence of pH. Holman (27) showed that crystalline soybean lipoxidase had a pH optimum near 9.4. Chemically, methyl linoleate is an ester, and its solubility is not affected by changes in pH, whereas sodium linoleate being a soap becomes increasingly more soluble with increase in pH. On the basis of these considerations, Smith (52) made a study of activity versus solubility of sodium linoleate. He observed that the increase in lipoxidase activity and in solubility of sodium linoleate were almost parallel. Using methyl linoleate, he found that the optimum pH was 6.5. This value is in agreement

with those obtained for lipoxidase in pea (49) 6.9, wheat (32) 6.5, and barley (16) 7.0.

Temperature. Lipoxidase is very active, even at low temperature. The energy of activation is 4.4 - 6.5 kcal./mole (32, 58), and Q_{10} is 1.6 between -1.5° to 18.5° C. (14). Although the enzyme is active at temperatures below freezing, it has been observed that in the frozen condition the rate of oxidation was only 1% of that in the liquid state.

Since sodium linoleate is frequently used as the substrate, the temperature as well as the pH may influence the solubility of the substrate, and consequently the rate. Smith (52) found that 25° C. was the most appropriate temperature at pH 6.5. Nevertheless, lipoxidase has often been studied at 30° C. (17, 32, 58), as well as at 20° C. (50). Secondary reactions become prevalent at higher temperatures, but at lower temperatures these reactions are negligible (27, 45).

Substrate. Plant lipoxidase oxidizes only linoleic, linolenic and arachidonic acids (29). They have, respectively, 2, 3 and 4 double bonds, each separated by a methylene group (12). A specific configuration of the molecule is required for lipoxidase catalysis. Thus it was shown by Privett et al. (45) that only the cis-cis configuration (see Fig. 1) can serve as substrate for lipoxidase. It is significant that cis-trans or trans-trans isomers are ineffective in view of the fact

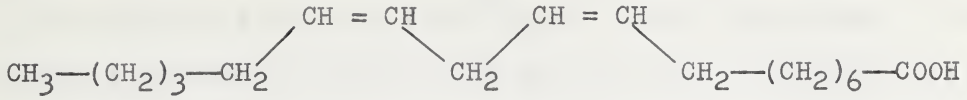


Fig. 1. Molecular structure of cis-cis-Octadeca-9,12-dienoic (linoleic) acid.

that the products have the cis-trans or trans-trans configuration (45). Oleic acid is the most common fatty acid in vegetable oils (6). It is also an 18-carbon unsaturated acid, but has only one double bond and is not an essential fatty acid. Oleic acid, as well as other unsaturated acids, can act as competitive inhibitors, but are not themselves oxidized (27, 39). This suggests that lipoxidase has an affinity for unsaturated groupings in fatty acids, but only compounds with a specific spatial arrangement of the double bonds can act as substrates for lipoxidase (29).

The study of optimum substrate concentration is hampered by a serious solubility problem (29). The soaps form emulsions in aqueous solutions, while the acid frequently is applied as an emulsion with Tween (59), gum arabic (36), gum ghatti (19), or Triton (32).

The reported Michaelis constants (Km) vary considerably. The most commonly quoted values are in the range, $1 - 3.5 \times 10^{-3}$ M (15, 16, 27). Tappel et al. (59) observed a Km of 2×10^{-5} M for soybean lipoxidase, and Irvine and Anderson

(32) reported a Km of 5×10^{-6} M for wheat lipoxidase. This extreme variation in Km values has not been explained, but, as Frehse (18) suggested, it appears to be unrelated to the method of measuring lipoxidase activity. Lipoxidase may be adversely affected by high concentrations of sodium linoleate (59). Since sodium linoleate is a surface-active compound, the deleterious effect may be due to a denaturation of the enzyme protein (2).

Oxygen requirement. The requirement for molecular oxygen seems to vary somewhat, depending upon experimental conditions. Holman (27) obtained maximum activity of crystalline soybean lipoxidase at 160 mm., and optimum at 40 mm. (partial pressure oxygen). Irvine and Anderson (32) report that above 40% oxygen the rate of reaction of wheat lipoxidase was independent of oxygen concentration. Franke and Frehse (16) concluded that at very low enzyme concentration oxygen does not become limiting, when the gas phase is air. As the enzyme concentration is increased, the oxygen requirement increases. Possibly the response to increasing oxygen concentration is related to diffusion of the oxygen (18).

Oxidation products. The products of the lipoxidase-catalyzed oxidation have been shown to consist primarily of conjugated diene hydroperoxides (45). These compounds are mainly cis-trans or trans-trans isomers of the acid (35, 45).

The configuration is significant, in view of the fact that trans-isomers of metabolites are utilized only sluggishly, if at all, in biological systems (47). Khan et al. (35), using displacement chromatography and infra-red spectroanalyses, found that trans-trans isomers predominated after autoxidation of linoleate catalyzed by ultra-violet light, visible light, or copper oleate. It was suggested that trans-trans isomers occur as ultimate secondary products, because they are thermodynamically more stable than cis-trans. Siddiqi and Tappel (49), as well as Privett et al. (45) found evidence for the production of carbonyl compounds (aldehydes and ketones) and polymerization during linoleate-lipoxidase oxidation. The enzyme appears to be directly involved in the formation of secondary products. Polymerization occurred only to the extent of 5%; 95% were monomers. The polymers, which consisted mainly of dimers, did not possess any carbonyl groups (45). The formation of secondary products may involve the participation of other factors than those necessary for conjugated diene formation (50). It may be significant that the products of autoxidized, lipoxidase, or copper-protein-catalyzed oxidation appeared to contain few carbonyl units (56, 57).

Khan et al. (35) recognized the presence of at least six distinct products from linoleate oxidation by a crude enzyme extract. The authors suggest the possible mediation of other enzymes in connection with lipoxidase catalysis. Moreover, they found four major products in the chlorophyll-light-catalyzed oxidation, but only two could be distinguished after

autoxidation catalyzed by copper-oleate, visible light, ultraviolet light, or in the dark at -10° C.

Tappel (56) observed that hemes catalyzed the breakdown of hydroperoxides. The products had fewer double bonds, and there was an increase in carbonyl compounds, which did not occur in the absence of hematin.

Siddiqi and Tappel (49) have postulated that cutin could be formed as a result of lipoxidase oxidation of unsaturated fats. The hydroperoxides could migrate along the cell wall until they reached the surface. In contact with oxygen of the air, these hydroperoxides could polymerize to form cutin. Hemes could perhaps be involved in such a process, as well as in the secondary reaction of linoleate-lipoxidase oxidation (50, 55). Only partly purified lipoxidase was used by the workers who reported carbonyl compounds accumulating during linoleate-lipoxidase oxidation (45, 49); hence the possibility of hemes being present cannot be excluded. It should be mentioned, however, that Holman observed an increase in carbonyl formation with increase in temperature using crystalline soybean lipoxidase (27).

Cofactors. Soybean lipoxidase does not require any metal, coenzyme or other cofactor for maximum activity (27). Kies (36) isolated a crystalline polypeptide which enhanced diene formation during linoleate oxidation by lipoxidase. However, a substance with a similar effect was present in gum arabic; hence the polypeptide was postulated as having influenced the

substrate rather than as having a direct effect on the enzyme.

Siddiqi and Tappel (49) have made a comprehensive study of pea lipoxidase. They concluded that none of the groups, metals, nucleotide coenzymes, or sulfhydryl, were involved in lipoxidase oxidation. However, they have later reported the participation of thiol groups in the oxidation of linoleate by urd bean lipoxidase (50).

Inhibitors. Many enzymic inhibitors have been employed to elucidate the possible mechanism and function of lipoxidase. Compounds which interfere with the action of a functional metal, as cyanide, azide, diethyldithiocarbamate, ethylenediaminetetraacetate, fluoride and pyrophosphate, have no effect on lipoxidase in pea, urd bean, mung bean, or peanut (49, 50). Similarly, no inhibition of 2,3',6-trichlorophenolindophenol oxidation by a linoleate-soybean-lipoxidase system was observed in the presence of cyanide or azide (20). However, Irvine and Anderson (33) reported a cyanide inhibition of wheat lipoxidase, but no inhibition occurred unless the enzyme was incubated with the cyanide for at least fifteen minutes. The per cent. inhibition varied with the amount of enzyme present in the reaction mixture.

Mapson and Moustafa (39) found a cyanide-sensitive and a cyanide-insensitive reaction in the coupled oxidation of linoleate-lipoxidase and glutathione. The cyanide-sensitive was a secondary reaction, and probably involved the oxidation of glutathione. Irvine and Winkler (31) found that cyanide in-

hibits the bleaching of pigments during dough-mixing. The authors postulate the participation of a cyanide-sensitive activator for the coupled reaction of fat oxidation and pigment bleaching.

The effect of thiol inhibitors on lipoxidase have in most instances been negative, but the recent observations by Siddiqi and Tappel (50) indicate that functional thiol groups may be present in certain lipoxidases. These workers observed that p-chloromercuribenzoate, iodoacetate, as well as maleate, exerted a pronounced inhibitory effect on urd bean lipoxidase. Lipoxidase in peanut, pea, soybean and wheat was not affected by these compounds (50). Glutathione reactivated the urd bean lipoxidase by 85%. This lipoxidase also was inhibited in the presence of catalytic amounts of Ag, Cu, and Hg ions. In addition to forming mercaptides, these ions also may combine with amino groups of proteins (50).

Antioxidants. The most universally effective inhibitors of lipoxidases are the antioxidants. Antioxidants function by supplying more easily extractable hydrogen atoms than those of the natural substrates (4). The remaining free radical of the antioxidant molecule is not active enough to function in linoleate oxidation; hence the inhibitory effect of the antioxidants on linoleate-lipoxidase oxidation (29).

Nordihydroguaiaretic acid (NDGA) is the most potent and most frequently employed antioxidant (Fig. 2) (50, 58, 59).

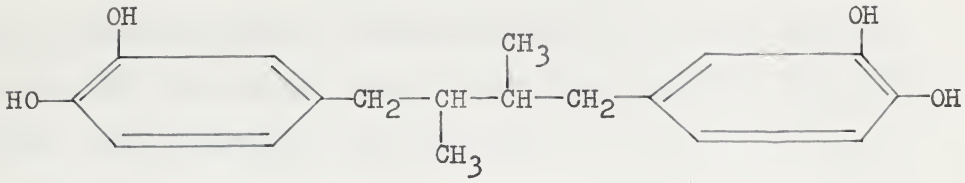


Fig. 2. Nordihydroguaiaretic acid (11).

According to Siddiqi and Tappel (50), the only resemblance between NDGA and lipoxidase substrates is that they both possess hydrogens which are easily abstracted. These workers (50) have shown that NDGA acts as a competitive inhibitor for linoleate oxidation by lipoxidase. Holman (29) suggested that antioxidants function by suppressing a chain reaction initiated by lipoxidase. Tappel et al. (59) maintain that such could not be the case because NDGA is oxidized even in the absence of net linoleate oxidation.

Many other antioxidants have been employed in lipoxidase studies. α-naphthol, propyl gallate, hydroquinone, pyrogallol and catechol depress oxygen uptake (50), diene formation (58), and bleaching of carotenoids (53).

Tocopherol. Tocopherol, or vitamin E, is a natural antioxidant. It is found in most green plants and in seeds (11). Carrot, wheat germ, corn, rice bran and soybean are especially rich in tocopherol. Corn, cottonseed and soybean oils also contain vitamin E. Much lower concentrations are found in olive oil, coconut oil, bananas and oranges (11).

This is particularly interesting in view of the observation that fats and oils of tropical plants are often lower in essential fatty acid content than those derived from plants grown in a temperate zone (6, 12).

Hickman (26) has expressed the view that α-tocopherol may regulate and maintain a suitable balance of "pro-oxidant" and "antioxidant" conditions in the tissue. It would, in effect, ensure the proper metabolism of the essential fatty acids. Indirectly, tocopherol may have a sparing effect on the essential fatty acids by virtue of its antioxidant properties (47).

Nevertheless, the function of tocopherol in plant metabolism is not known. It acts as a mild antioxidant in linoleate-lipoxidase oxidation in vitro (50, 58). Vitamin E may, then, be one of a number of controlling factors for plant lipoxidase in vivo.

Coupled oxidation. Linoleate-lipoxidase, and unsaturated-fat-lipoxidase, oxidation have been shown to be coupled to pigment oxidation (37, 53). During this process, the pigments are bleached and "destroyed" (53). Besides carotenoids, hemes and chlorophyll are also bleached during the oxidation (53).

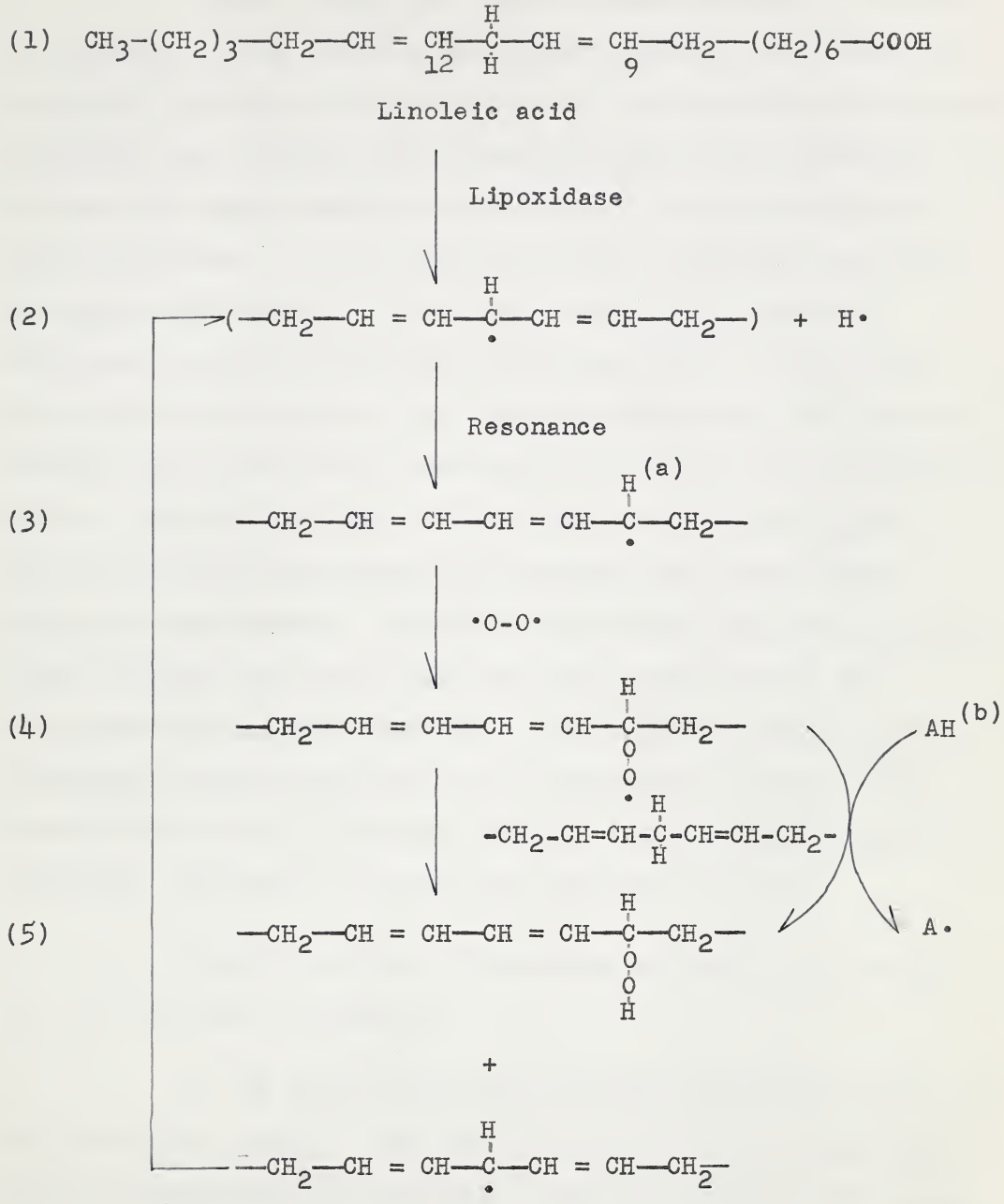
Fatty acid peroxides alone do not decolorize pigments (31). Strain (53) has claimed that an intermediate oxidative product is responsible for the pigment oxidation. On the basis of their results, Irvine and Winkler (31) have proposed

a sequence for xanthophyll destruction in macaroni dough. They assume that lipoxidase and oxygen (or linoleate) may exist in a combined form. During the mixing of the dough, all three factors are brought into contact, and an intermediate peroxide is formed which oxidizes the pigments.

Certain dyes (p-phenylenediamine, dihydroxyphenylalanine (dopa), and 2,3',6-trichlorophenolindophenol) also can be oxidized during linoleate-lipoxidase oxidation (20, 53). Also, glutathione (38) and ascorbate (53) may be oxidized during unsaturated fat oxidation.

Reaction mechanisms. The mechanism whereby lipoxidase catalyzes the peroxidation of essential fatty acids has not been clarified. Autoxidation proceeds by a free radical chain reaction (4). It was proposed by Holman (27) that lipoxidase might initiate a chain reaction by creating a free radical from linoleate (16). The reaction could then proceed autocatalytically. A modification of the proposed scheme (27) is outlined in Figure 3.

The free radicals may be formed continually. If antioxidants were present, the peroxyl radical (4) could extract a hydrogen from an antioxidant molecule (b). Since the antioxidant-free radical is not sufficiently active to extract a hydrogen atom from linoleic acid, the rate of linoleate oxidation will be depressed.



(a) The free radical could be on C_{13} or C_{11}.

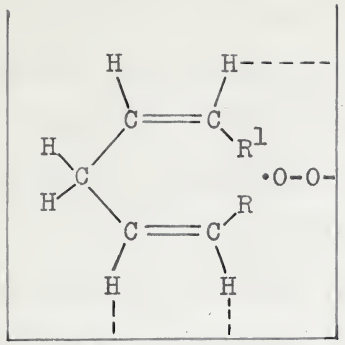
(b) Antioxidant molecule.

Fig. 3. Oxidation of linoleic acid by lipoxidase (29).

Tappel et al. (59) made a thorough study of soybean lipoxidase. They maintain that under suitable conditions the enzyme is involved in the oxidation of each molecule of linoleate. Moreover, they observed that linoleate-lipoxidase oxidation follows the enzyme kinetics and not that for autoxidation and chain reactions. It is well-known that a substrate must have the cis-configuration of the double bonds and an isolated methylene group for lipoxidase catalysis (45). It has also been shown that products from lipoxidase catalysis of linoleate exhibit optical activity, whereas the products from autoxidation show no optical activity. The latter observations suggest a specific spatial arrangement of substrate and enzyme before catalysis can proceed. On the basis of the foregoing considerations, Siddiqi and Tappel (50) have presented a model for linoleate-lipoxidase oxidation. These workers claim that the lipoxidase protein may function as an electron sink and thus momentarily retain an electron from the α-methylene group of linoleate, ultimately allowing the oxidation to occur.

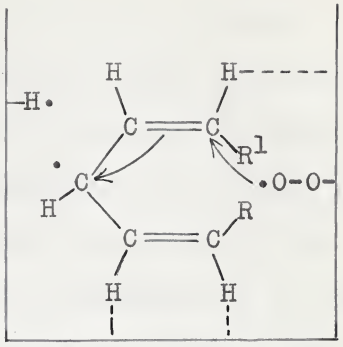
A modification of the scheme by Siddiqi and Tappel (50) is outlined in Figure 4.

In the first step (a), an enzyme substrate complex is formed with oxygen. The second step (b) is the formation of the α-methylene-free radical. That may involve the transfer of a hydrogen atom or the abstraction of an electron by the enzyme. The proton would pass into the medium. In the next



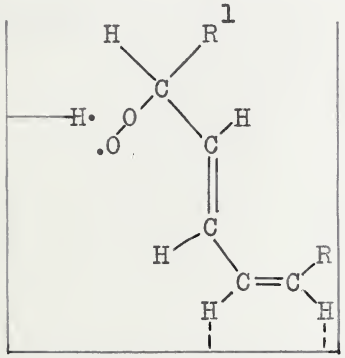
(a) Enzyme-linoleate-O_2 complex.

$-(H^+ + e^-)$ or $H\cdot$



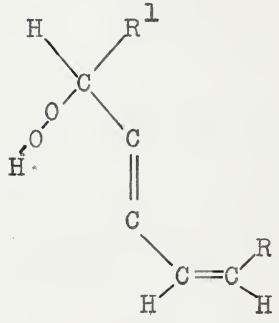
(b) α-methylene-free radical.

Isomerization



(c) Conjugated hydroperoxide-free radical.

$+(H^+ + e^-)$ or $H\cdot$



(d) Cis-trans conjugated diene hydroperoxide.

R^1 = COOH - $(CH_2)_7$—

R = CH_3 - $(CH_2)_4$—

Fig. 4. Oxidation of cis-cis-octadeca-9,12-dienoic acid by lipoxidase.

step (c), the double bonds isomerize, resonance occurs, and oxygen immediately attacks the free radical in such a manner that an asymmetric center is created. The peroxyl radical now may receive a hydrogen atom or an electron from the enzyme

and form the hydroperoxide. Alternatively, the peroxyl radical could acquire a hydrogen atom from another molecule of linoleate or an antioxidant. The cooxidation of antioxidants, carotenoids, and other compounds may be brought about by the abstraction of hydrogen from these compounds by the peroxyl-free radical (c). The outlined mechanism is in agreement with known characteristics of lipoxidases (50). The recent report (50) of active sulfhydryl groups on urd bean lipoxidase may suggest a special kind of lipoxidase in those plants.

MATERIALS AND METHODS

I. Preparation of Enzymes

(a) Sunflower Meal Lipoxidase (Em)

The seeds, consisting of a mixture of several varieties of sunflower, were finely ground and extracted with diethyl ether or petroleum ether. In order to prevent heat inactivation of the enzyme, oil extraction was performed at room temperature. The meal was packed firmly in a glass cylinder, and solvent allowed to percolate down through the meal until all apparent ether-soluble material had been removed. After extraction, the meal was removed from the cylinder and placed in a tray at 20° - 25° C. in air until the ether had evaporated. Similarly, the ether was evaporated from the extracted oil, and the defatted meal as well as the oil were stored at 2° C. until used.

The enzyme extract was prepared by suspending the defatted meal in dilute buffer. Six grams of meal were ground in a mortar with 30 ml. 0.01 M potassium phosphate buffer, pH 7.5, at 0° C., and the slurry was centrifuged for 10 - 15 min. at 20,000 x g. The clear, yellowish-brown supernatant, containing the enzyme, had a nitrogen content of 3.0 - 3.5 mg./ml. This enzyme was used immediately.

(b) Sunflower Seedling Lipoxidase (Es)

The seedlings were obtained by germinating seeds in moist vermiculite (heat-treated mica), at constant temperature in the dark.

To study relative lipoxidase activity during germination, the following procedure was used: The seedlings were washed thoroughly and ground in a Servall high-speed omnimixer at half-maximum speed. The seedlings (10 - 25 gm.) were homogenized in 50 ml. 0.1 M phosphate buffer, pH 7.5, at 0° - 2° C. The homogenate was filtered through cheesecloth and centrifuged for 10 - 15 min. at 20,000 x g. The clear, yellow supernatant, containing from 0.4 to 1.3 mg. N/ml., was used as the source of the enzyme.

In cell fraction studies, the homogenate was prepared by grinding the seedlings (seed-coats removed) in a solution containing 0.44 M sucrose and 0.1 M potassium phosphate buffer, pH 7.5. The fractionation procedure is outlined in Figure 5. All operations were performed at 0° - 2° C. A Servall super-speed angle centrifuge, Type SS-1, was used for speeds up to 25,000 x g. For the sedimentation of the microsomal fraction, a Spinco ultra-centrifuge, Model E (rotor K), was used.

(c) Partial Purification of Sunflower Seedling Lipoxidase

Acetone powders were prepared from the crude homogenate (Fig. 5). Two hundred ml. crude homogenate (no sucrose)

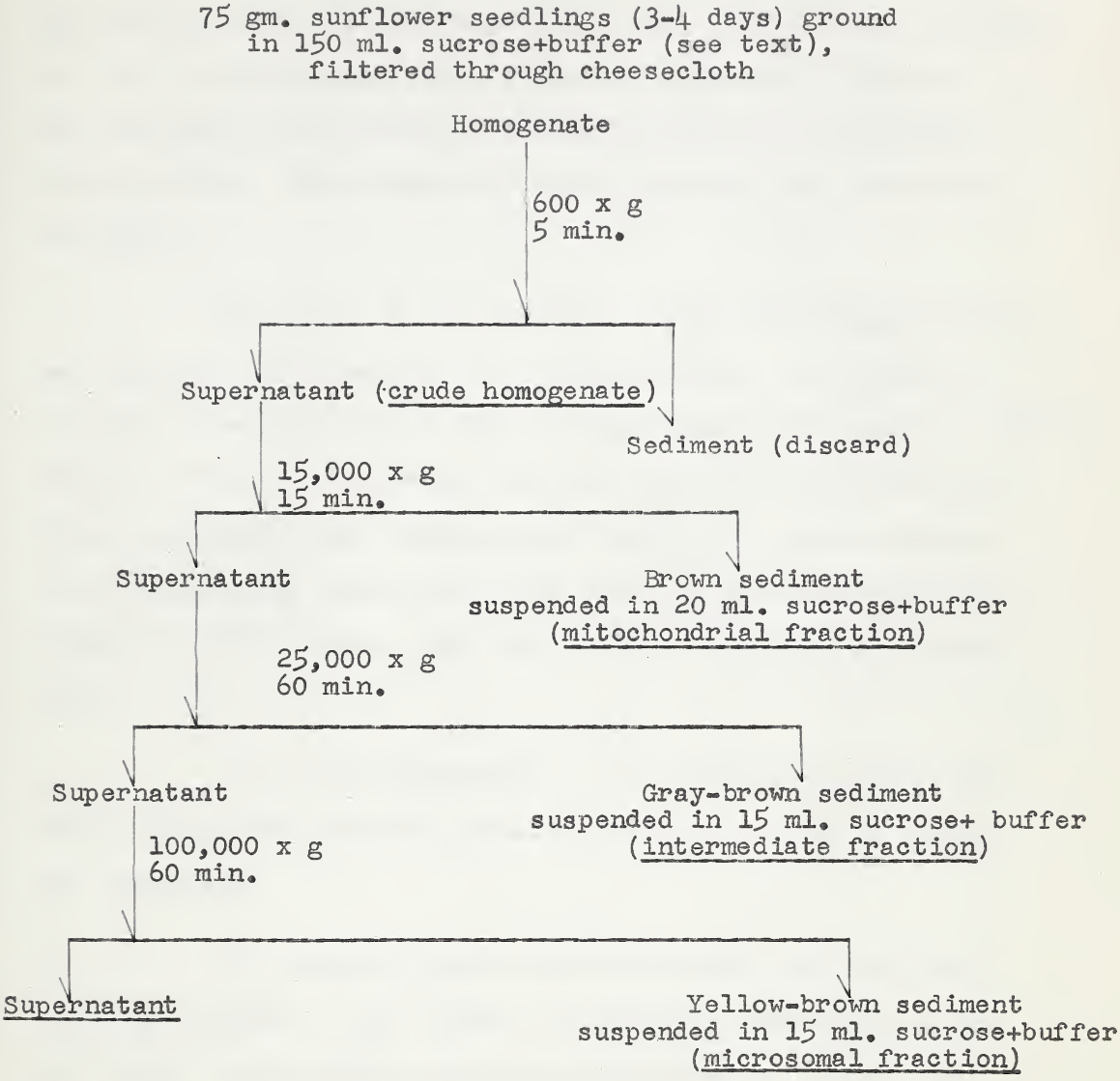


Fig. 5. Fractionation of sunflower seedling homogenate.

were added with vigorous stirring to 2 l. acetone at -20° C. The mixture was adjusted to pH 4.5 - 5.0, filtered on a Buchner, and the residue washed three times with acetone. Suction was continued in a stream of nitrogen until the acetone had been removed. The light-tan powder was kept in a desiccator at -20° C.

For most of the kinetic studies, the enzyme extract was prepared by suspending the acetone powder in a mixture of 2 parts of neutralized 1% (W/V) deoxycholate and 1 part 2×10^{-2} M $KHCO_3$ at 0° C. The powder and the solution were placed in a glass homogenizer and "homogenized" gently to avoid foaming. After 30 minutes' extraction, the solution was centrifuged at 20,000 x g for 15 min. The supernatant contained the enzyme (Ep).

In a few experiments, dilute phosphate buffer was used, rather than deoxycholate-bicarbonate, but the procedure was identical.

The enzyme preparation was further purified with ammonium sulfate. Two volumes of saturated ammonium sulfate were added to the clear supernatant obtained by deoxycholate-bicarbonate extraction. The ammonium-sulfate precipitate was centrifuged at 10,000 x g and the precipitate dissolved in 5×10^{-2} M potassium phosphate buffer, pH 7.5 (E_{AS}).

For spectrophotometric analyses, the E_{AS} was dialyzed against 600 vol. of distilled, demineralized water for 24 hr.

(d) Preparation of Flax, Rape, Wheat Germ, and Soybean Lipoxidase Extracts

Flax, rape and wheat germ extracts were prepared from defatted meals as outlined for sunflower meal lipoxidase. An acetone powder of soybean seedlings was used as the source of soybean lipoxidase. It was prepared as outlined for sunflower lipoxidase.

II. Determinations

(a) Per Cent. Total Oil and Free Fatty Acids

Total oil and free fatty acids were determined by the methods recommended by the American Oil Chemists' Society (1).

(b) Nitrogen Determination

Total nitrogen was determined by the Microkjeldahl method, as outlined in the publication of the Association of Official Agricultural Chemists (3), except that hydrogen peroxide was added to hasten the digestive process (22).

(c) Lipoxidase Activity

The enzyme preparations were incubated in the standard Warburg monometric apparatus (60). Normally, the reaction mixture contained K linoleate, enzyme extract, phosphate buffer, pH 7.4 or 7.0, in a total volume of 2.1 ml. The center

well contained 0.1 ml. of 20% KOH. After temperature equilibration, the enzyme or the substrate was added from the side arm. The samples were incubated at 20° C. for 20 min. if the gas phase was oxygen, and 60 min. if the gas phase was air. The shaking rate was 120 oscillations per minute.

Spectrophotometric analysis. For the spectrophotometric analyses of the products of oxidation, the reaction mixture contained 20 micromoles K linoleate, 1.3 ml. 0.2 M K-phosphate buffer, and 0.6 ml. enzyme extract (E_{AS} dialyzed). The final volume was 2.0 ml., and the pH 7.0. Immediately after the oxidation was completed, 0.2 ml. of the reaction mixture was added to 5 ml. 95% ethanol. A 1 ml. aliquot of this mixture was added to 20 ml. 60% ethanol. The optical density was then measured in the range 220 - 300 mμ on a Beckman DU spectrophotometer.

III. Substrates

Linoleic and oleic acids were purified preparations obtained from Fisher Scientific Co. Chemically pure ethyl linoleate was a product of the Hormel Institute. The acids were neutralized with KOH and kept at -20° C.

RESULTS AND DISCUSSION

I. Lipoxidase in Sunflower Seeds (Em)

In preliminary studies of lipoxidase in seeds of various plants, it was observed that lipoxidase was quite active in flax seeds and wheat germ, less active in sunflower, and totally inactive in rape seeds. These results agreed with those of Franke and Frehse (17) except in the case of sunflower, which they had found to be lipoxidase-negative.

Sunflower seed lipoxidase (Em) was very unstable after extraction from the defatted meal. Consequently, this enzyme was freshly prepared for each determination. Dialysis against 0.05 M phosphate at pH 7.4 for 16 hr. at 1° - 2° C. resulted in complete loss of activity. The instability may have been due to the presence of soluble, destructive enzymes in the crude extract. Rapid darkening of the extract also indicated a high polyphenol oxidase activity.

In preliminary experiments, Em required an induction period similar to that observed in autoxidation. However, it was later found that under conditions of adequate substrate, oxygen, pH, temperature, and especially enzyme concentration, this induction period could be eliminated.

The extracted sunflower oil had a distinct effect on linoleate oxidation by sunflower lipoxidase (Em). Additions

of two drops of oil to the reaction mixture stimulated the initial rate of oxygen consumption (Fig. 6 - I), suggesting that addition of substrates inherent in the oil could have been responsible; but increasing the concentration of K linoleate resulted in a decrease in the initial rate.

By emulsifying linoleic acid with 0.2% bile (bile-linoleate), it was possible to imitate the effect of oil on initial rate (Fig. 6 - II). Possibly the oil may have had a non-specific effect on the substrate.

Figure 6 also illustrates the influence of vegetable oil on the total oxidation of linoleic acid. After a short period of high activity, lipoxidase oxidation in the presence of oil ceased almost entirely. The enzyme could have been inhibited by adsorption of the protein on the fat globules, with consequent denaturation, but increasing the amount of oil did not increase its effect on total oxidation. An obvious explanation could have been the presence of natural antioxidants in the oil. Sunflower oil does contain a small amount of tocopherol (11), but 5×10^{-3} M α-tocopherol emulsified in 0.2% bile had no inhibitory effect on lipoxidase oxidation. Moreover, the presence of antioxidants reduces the rate, as well as the total oxidation; consequently, antioxidants could not have been entirely responsible.

Since fatty acid hydroperoxides have a deleterious effect on certain enzymes (5), the hydroperoxides inherent in

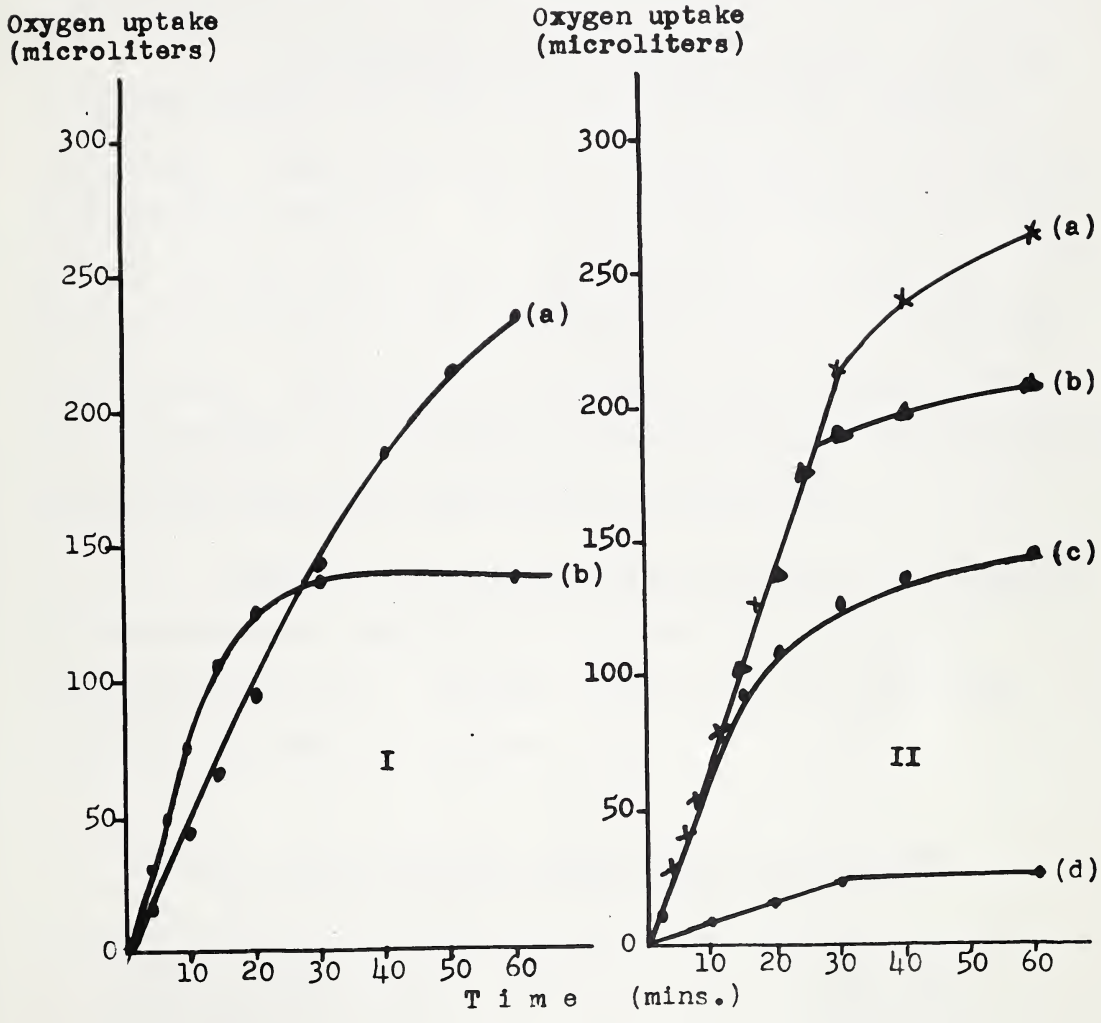


Fig. 6. Influence of vegetable oil on linoleate-lipoxidase oxidation.

Reaction mixtures:

I. (a) K linoleate *
(b) K linoleate + 0.05 ml. sunflower oil *

II. (a) bile-linoleate + 5×10^{-3} M α-tocopherol *
(b) bile-linoleate *
(c) bile-linoleate + 0.05 ml. bile-oil *
(d) 0.05 ml. bile-oil *

* + Enzyme + phosphate buffer, pH 7.5
(linoleate concentration: 15 micromoles).

the oil might also have influenced lipoxidase. However, total oxidation was not affected by the amount nor by the age of the oil. Pre-heating the oil at 100°C. for 10 min. did not diminish its effect on linoleate-lipoxidase oxidation.

It is remarkable that, although sunflower oil contains 58% linoleic acid (6), it was oxidized very slowly by lipoxidase. Emulsifying the oil with bile salt did not appreciably increase oxidation. It was noted that linoleic acid, as well as the free fatty acids from saponified sunflower oil, when present as droplets in the reaction mixture were oxidized very rapidly. Therefore, it is possible that linoleic acid as a constituent of glycerides or of phospholipids was less readily oxidized by lipoxidase than as free linoleic acid.

Addition of rape-seed oil and flax-seed oil similarly depressed total oxidation of linoleic acid by sunflower lipoxidase (Table 1). However, rape-seed oil was much less effective than flax or sunflower oil.

Table 1. Inhibition of oxygen uptake by vegetable oils during linoleate-lipoxidase oxidation*.

Vegetable oil	% reduction
Rape oil	23
Sunflower oil	44
Flax oil	50

* Experimental conditions as in Fig. 6.

Franke and Frehse (17) report a petroleum-ether-soluble lipoxidase inhibitor present in barley seeds, which are lipoxidase-active. Although tocopherol was present in barley seeds, it was not shown by these workers that this compound was identical to the inhibitor. Franke and Frehse (16) also observed that oats were lipoxidase-negative shortly after harvest; a phenomenon they associated with the presence of inhibitors (possibly antioxidants). It is conceivable that "lipoxidase-active" seeds contain lipid soluble-substances which can curtail excessive peroxidation of essential fatty acids. This would account, in part, for the very low rate of oxidation of sunflower and flax oil (80% linoleic and linolenic acids, (6)) by lipoxidase.

However, a detailed study of the vegetable oils and their inhibitory effect on lipoxidase oxidation of linoleate would be essential before any valid conclusions could be drawn.

II. Lipoxidase Activity During Germination of Sunflower Seeds

The results of an investigation on the relative lipoxidase activity of the supernatant fraction (20,000 x g) during germination of sunflower seeds are presented in Figure 7.

The increase in rate of lipoxidase activity is very rapid during the initial stages of germination, and a high rate is maintained for a few days, after which it gradually decreases. These results differ somewhat from those reported for other

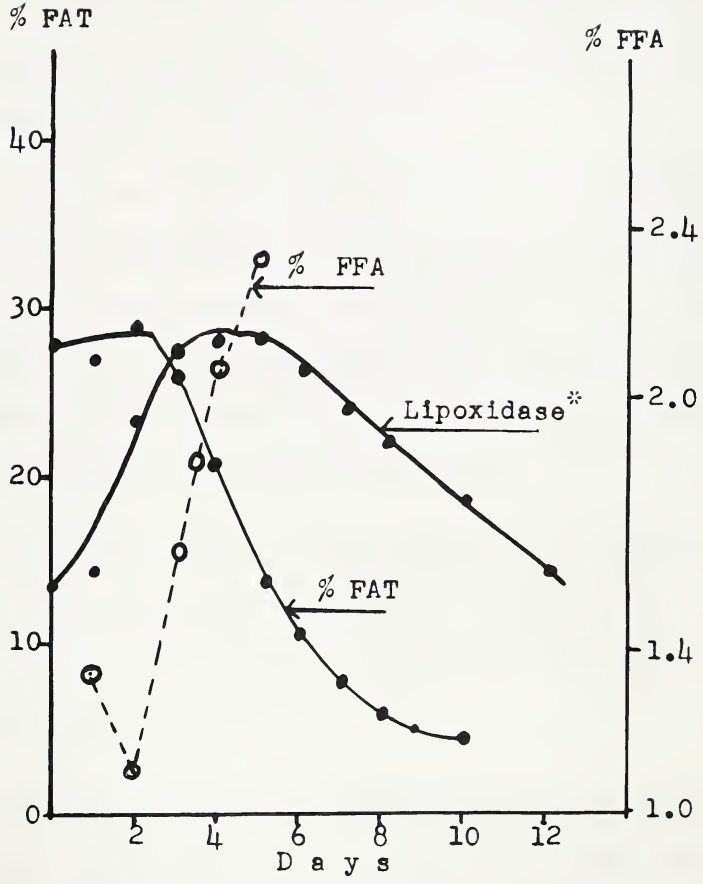


Fig. 7. Lipoxidase activity*, per cent. oil, and per cent. free fatty acids during germination of sunflower seeds.

* µl O_2/min.
Reaction mixture: contained enzyme + 15 µmoles K linoleate + phosphate buffer, pH 7.5.

plants. Soybean lipoxidase activity decreases rapidly after the second day (28). With corn lipoxidase (19), there is a sharp increase in activity on the second day, followed by an equally sharp decrease on the fourth day. By the sixth day, the activity is practically absent. The differences in relative activity of lipoxidases of different plant species may reflect differences in conditions during germination and/or in the enzymic complement of the seedlings.

As indicated in Figure 7, there is an apparent slight increase in total oil during the early stages of germination. It might be due to preferential utilization of other compounds, such as sugars (6). After the second day, the fats are hydrolyzed (increase in free fatty acids, Fig. 7), and rapidly depleted or transformed into other products.

The iodine number of the extracted oil from the seedlings was not determined. However, neither Holman (28) nor Fucuba (21) found any correlation between iodine number and lipoxidase activity.

From the results of this investigation and those of Holman (28) and others (16, 19, 39), it would appear that lipoxidase may have some vital function during germination. Since it appears that high lipoxidase activity and depletion of fat reserves occur simultaneously, it is possible that lipoxidase has a significant metabolic function in the mobilization of lipids in germinating sunflower seeds.

III. Sunflower Seedling Lipoxidase in Cell Fractions

Sunflower lipoxidase was present in all the fractions (Table 2). The enzyme is apparently associated with the particulates as well as with the soluble portion of the homogenate.

Table 2. Linoleate oxidation by various cell fractions of sunflower seedlings.

Fraction	% N	In air * µl. O_2/min.	In O_2 * µl. O_2/min.
Crude homogenate, 600 x g	100	14.3	58.5
Mitochondrial, 15,000 x g	5.0	13.8	57.0
Intermediate, 25,000 x g	3.8	13.5	59.0
Microsomal, 100,000 x g	5.5	15.0	61.5
Supernatant	84.8	13.3	57.0

* Reaction mixture contained: 15 µmoles K linoleate + phosphate buffer, pH 7.5 + enzyme extract.

Experiments were conducted in an attempt to determine the nature and degree of association of lipoxidase with the particulate structures of the cell. Crude homogenate was treated with ammonium sulfate. The insoluble portion was sedimented and re-dissolved in dilute buffer. After dialysis for 42 hr. against distilled, demineralized water, the extract

was centrifuged. The sediment (particulate matter) oxidized linoleate at the same rate as the supernatant. Dialysis against large volumes of distilled, demineralized water should have brought about swelling and bursting of the particulates, releasing any soluble enzymes which ultimately would be limited to the supernatant portion.

Further evidence that lipoxidase may be closely associated with the particulate structures is presented in Table 3. A sediment obtained at 25,000 x g from the crude homogenate was dispersed in 2% (W/V) deoxycholate. Deoxycholate (DOC) promotes the solubility of proteins (10).

Table 3. The effect of deoxycholate treatment on lipoxidase activity in cell particulates of sunflower seedlings.

Fraction	In O_2
	µl. O_2/ min.
Crude homogenate, 600 x g	67.0
Sediment, 25,000 x g	66.5
Supernatant, 25,000 x g	54.0
Sediment after DOC treatment	71.0
Supernatant of DOC treatment	74.0

Approximately 60% of the nitrogen was removed from the sediment by this treatment. Thus it is surprising that the activity of the sediment after deoxycholate treatment is so high. It may, in part, be due to a stabilizing effect of deoxycholate on the substrate and/or on the enzyme. Deoxycholate is a component of bile, which aids emulsification and lipolytic activity in the digestive system of mammals. Therefore, the substrate may be more readily available in the presence of deoxycholate, thereby producing an increase in the rate of oxidation. Precipitation and resuspension in acetone at -20° C. also failed to remove lipoxidase completely from particulates.

These results suggest that sunflower lipoxidase is, in part, linked to the cell particulate structures. Goodwin and Waygood (24) showed that barley lipoxidase was associated with the particulates, but that pea (39) and corn (19) particulate fractions did not contain an active lipoxidase. Fritz and Beevers (19), on the basis of their results, proposed that lipoxidase *in vivo* may be intimately associated with its natural substrate, which was indigenous with the particulate fraction. By mechanical injury, such as grinding and centrifugation, or by enzymic action (24), this relationship is distorted and lipoxidase is separated from its natural habitat -- the particulates. Although these considerations may not satisfactorily explain why sunflower lipoxidase was so prevalent in all of the cell fractions, it was evident that some of the active lipoxidase could be re-

moved, and that a fraction was intimately associated with the particulate structures.

IV. Kinetics of Sunflower Seedling Lipoxidase (Ep and E_{AS})

(a) The Influence of Substrate

Sunflower lipoxidase oxidized K linoleate very rapidly, but ethyl linoleate was a poor substrate and K oleate was inert. Under optimum conditions, the total oxidation of K linoleate was 75% to 80% of the theoretical maximum (1 M O_2/M linoleic acid).

As may be seen in Figure 8, oxygen was a limiting factor unless the partial pressure of oxygen was greater than that of air. The rate of oxygen uptake was measured for a series of substrate concentrations varying from 2.0×10^{-2} to 2.5×10^{-3} M (5 - 40 µmoles), in an atmosphere of air and in 100% oxygen (Figure 8).

The results obtained with oxygen as the gas phase were analyzed by the method of Lineweaver and Burk (cit. 42). This involves rearranging of the Michaelis-Menten equation and plotting 1/v against 1/s, where

1/v is the reciprocal of the rate, and

1/s is the reciprocal of the substrate concentration.

The slope of the line is Km/V and the ordinate intercept is 1/V, where

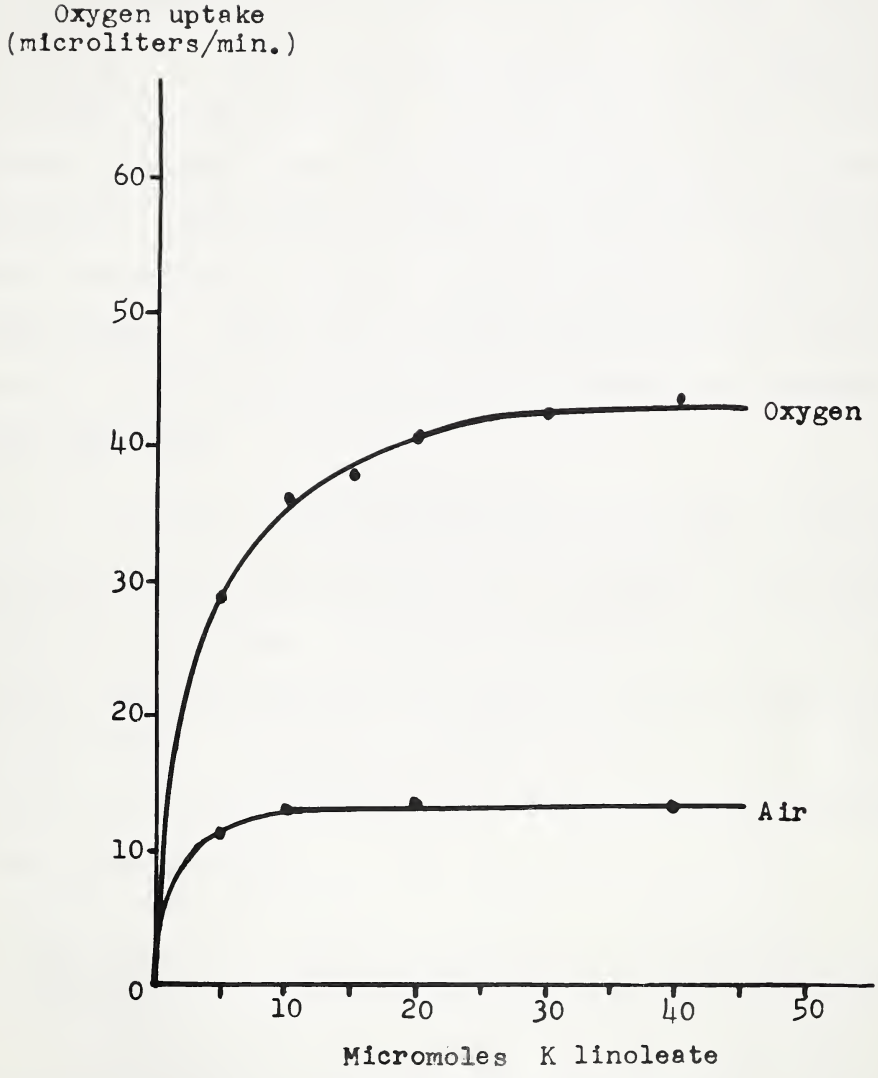


Fig. 8. Rate of oxygen uptake at various concentrations of K linoleate, at 20° C. in air and in oxygen. (Enzyme: E_{AS}, 0.15 mg. N).

Km is the Michaelis-Menten constant, and

V is the rate at maximum activity.

Knowing 1/V and Km/V, Km is readily calculated. The Km for sunflower lipoxidase was found to be 1.64×10^{-3} M. Reported values of Km for lipoxidases from various sources range from 1.35×10^{-3} to 5.0×10^{-6} M. The Km value of 1.64×10^{-3} M is therefore slightly higher than that found by other workers for lipoxidases. The method of measuring the activity may have limited the minimum substrate concentration that could be used in the assays. Unless the substrate concentration was kept above 2.5×10^{-3} M, the substrate was oxidized too rapidly to measure the rate.

In the course of these investigations, it was also noted that higher concentrations of K linoleate (soaps) depressed the rate of oxidation. A similar inhibiting effect by excess substrate has been observed with barley lipodehydrogenase (17). Since detergents are known to denature proteins (2), it is possible that excess substrate may have resulted in denaturation of the enzyme protein.

(b) <u>The Effects of Enzyme Concentration and Per Cent. Oxygen</u>

The studies of sunflower lipoxidase indicated that it generally followed normal enzyme kinetics. It was evident, however, that oxygen plays a very important role in the reaction. There is a linear relationship between oxygen concentration and rate of linoleate oxidation when the enzyme and substrate are not limiting the reaction

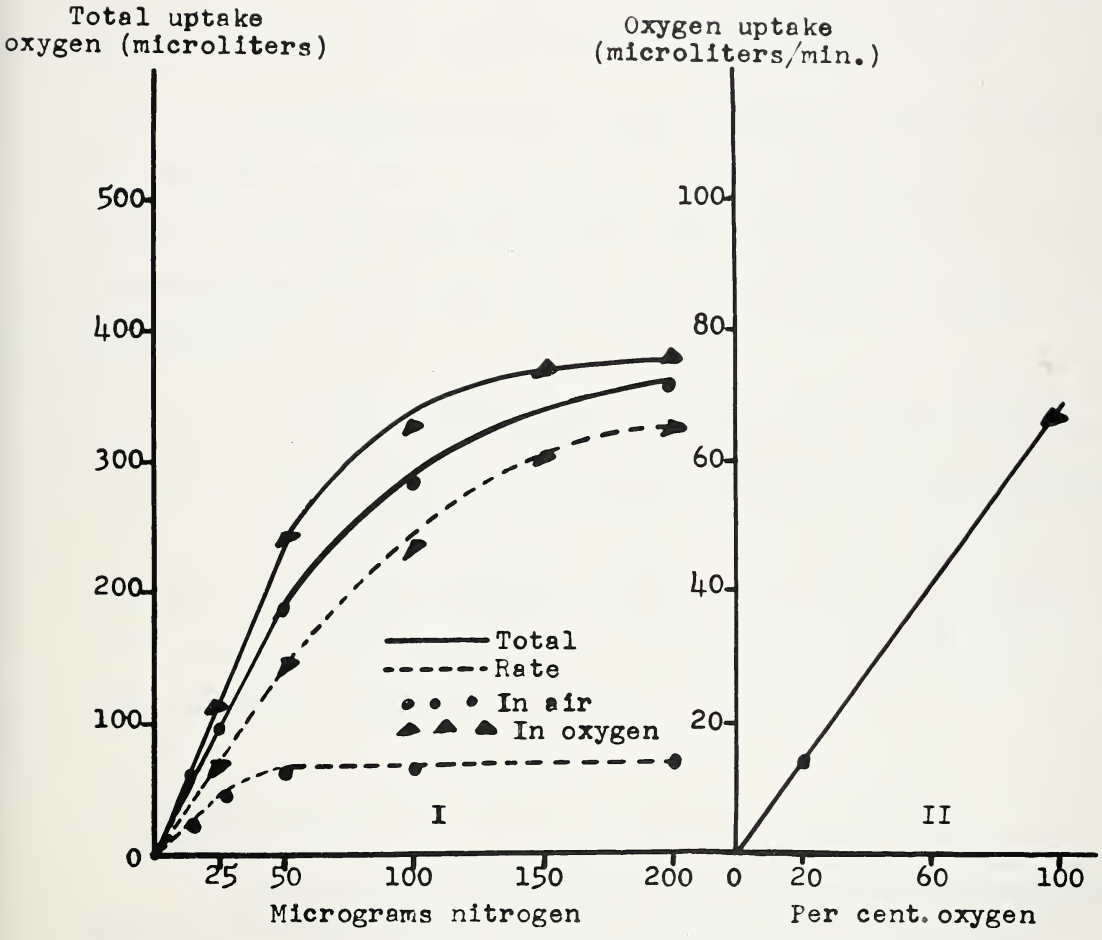


Fig. 9. Effect of enzyme concentration and of oxygen concentration on the rate and the total uptake of oxygen during linoleate-lipoxidase oxidation.

Reaction mixture: contained 20 μmoles K linoleate, phosphate buffer, and enzyme (E_{AS}).

(Figure 9 - II). This direct relationship may, in part, be a diffusion phenomenon. As indicated in Figure 9 - I, the initial rate was proportional to enzyme concentration at both oxygen levels, at low enzyme concentrations. Oxygen limited the rate. The lower total oxidation in air may infer that some of the enzyme had been inactivated as a result of the longer incubation period required when the reaction is carried out in air.

Theoretically, the total oxidation ought eventually to have been the same, regardless of the prevailing amount of enzyme. The data revealed that, over a limited range of enzyme concentrations, the total uptake was proportional to enzyme concentration.

These results agree in general with those obtained by Franke et al. (15). The findings suggest that the products could act as competitive inhibitors, or that linoleate and lipoxidase may form a complex which dissociates very slowly. Tappel et al. (59) visualize a linoleate-lipoxidase complex in which linoleate could function as a "coenzyme."

(c) pH Optimum

The activity was determined with air as the gas phase. Buffers (citrate-K phosphate, K_1 - K_2 phosphate, and borax-boric acid) were selected to cover the pH range, 3.65 to 9.3. The pH optimum was 6.8 (Figure 10).

Since potassium linoleate is not soluble below pH 9.2, it is evident that substrate solubility does not determine the optimum pH in the present system. Perhaps lipoxidase

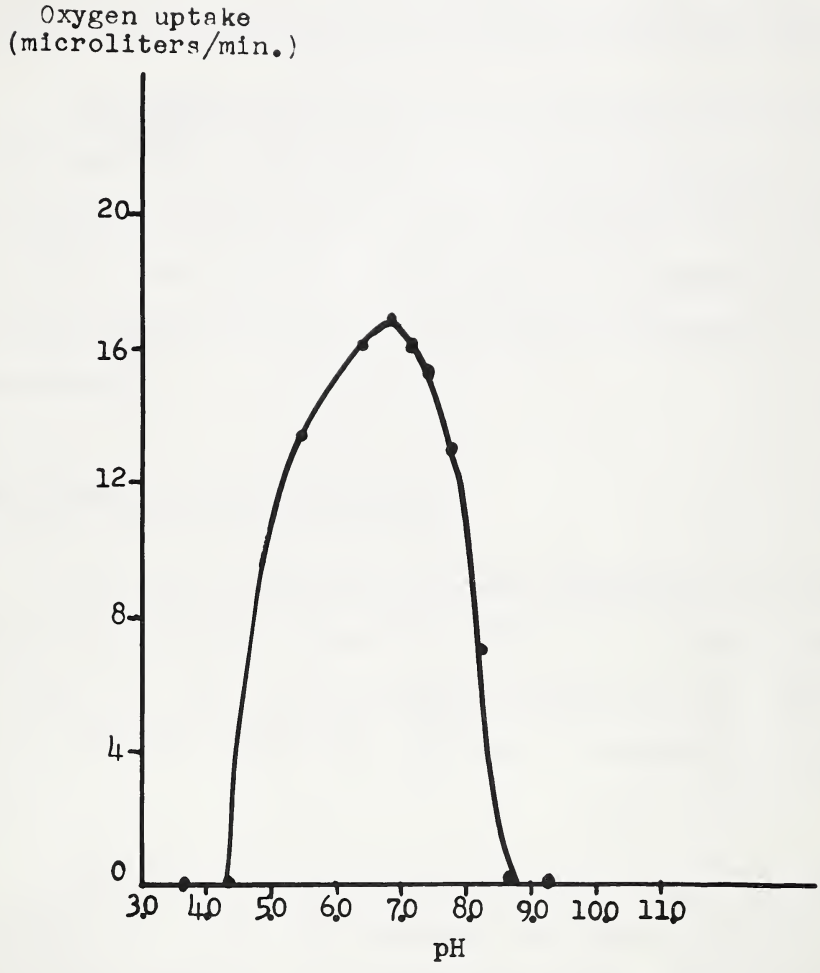


Fig. 10. The effect of hydrogen-ion concentration on linoleate oxidation by sunflower lipoxidase.

itself is also affected by changes in hydrogen-ion concentrations. It was found that some lipoxidase activity remained after dialysis at pH 9.7. Therefore, there may be reason to believe that the observed lack of activity at pH levels above 9.0 is, in part, due to denaturation of lipoxidase by the substrate.

The optimum pH of 6.8 is in agreement with that reported for most other lipoxidases. However, investigations in this laboratory showed that soybean lipoxidase was equally active at pH 7.6 and pH 9.6. This agrees in general with the work of Holman (27) who used a crystalline preparation of soybean lipoxidase.

(d) The Influence of Temperature

Sunflower lipoxidase was not appreciably affected by changes in temperature from 5° to 30° C. if the gas phase was air (Fig. 11 - I). In an oxygen atmosphere, the increase in rate of oxidation per 10° C. increase in temperature (Q_{10}) was 1.25.

The effect of temperature on total oxygen uptake is shown in Figure 11 - II. The increase in total oxidation at 20° - 30° C. is, perhaps, associated with secondary reactions which could be catalyzed by other enzymes (29). At 40° - 43° C., the enzyme proteins may have been partly denatured by the substrate (soap) in conjunction with temperature increases.

The energy of activation (E) was determined for the reaction in oxygen by plotting $\log_{10} k$ against $1/T$. The slope

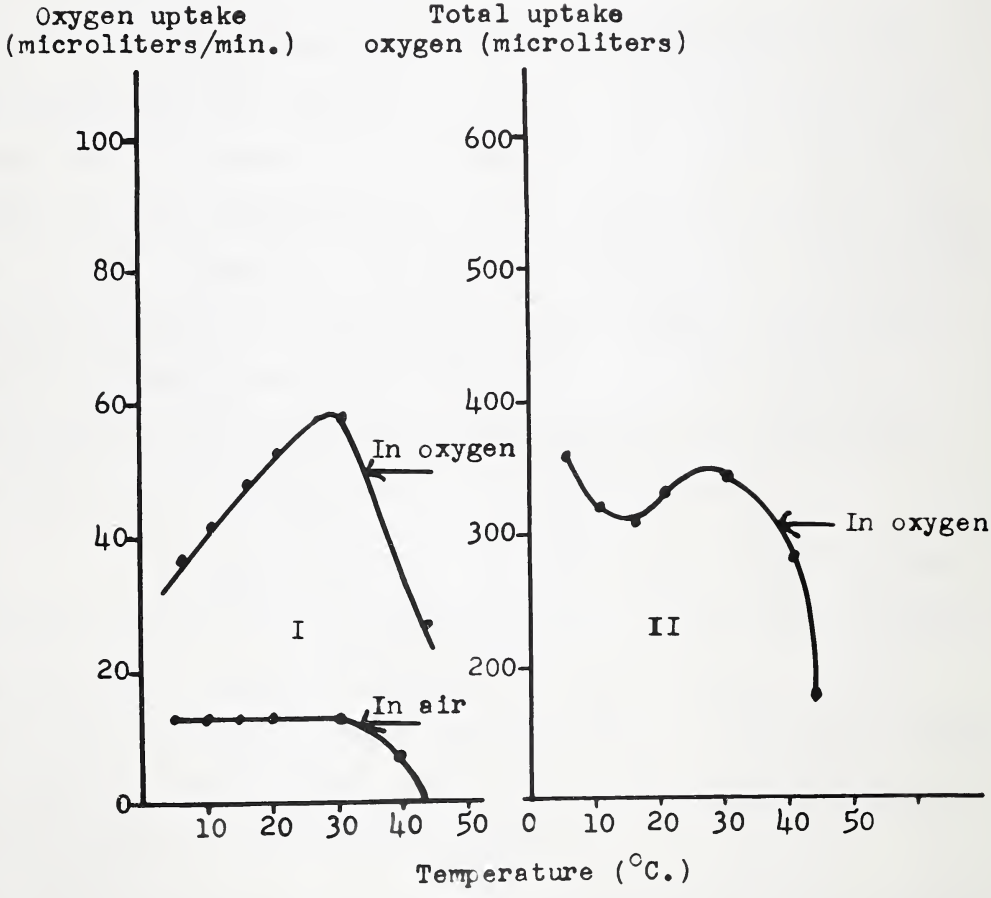


Fig. 11. Rates (I) and total oxygen uptake (II) during linoleate-lipoxidase oxidation at various temperatures.

Reaction mixture: See Fig. 9.

of the straight line is -0.219 E (42). E was 3.5 kcal./mole. This value is lower than the value of 4.3 kcal./mole obtained by Tappel (58) for soybean lipoxidase, but indicates that sunflower lipoxidase is very active even at low temperatures.

(e) Conjugated Diene Formation

Ultra-violet spectrophotometric analyses of the lipoxidase oxidized linoleate indicated that the primary compounds were conjugated dienes (Fig. 12 - I). Since the substrate was not chemically pure, some of the acid may have been autoxidized. However, analyses of substrate alone after additional incubation in oxygen for 20 min. contained very small amounts of conjugated dienes (Fig. 12 - I). Therefore, autoxidized fatty acids (hydroperoxides) did not contribute quantitatively to diene content in these studies.

In Figure 12 - II, the total oxygen uptake by the reaction mixture before spectrophotometric analysis is plotted against total amounts of conjugated dienes (OD_{234}). Since these data are from a single determination, it may not be valid to conclude that a direct relationship exists between total oxygen uptake and total diene formation.

The majority of the oxidation products were probably hydroperoxides, or perhaps 9 or 13 hydroxyl derivatives of linoleic acid (35).

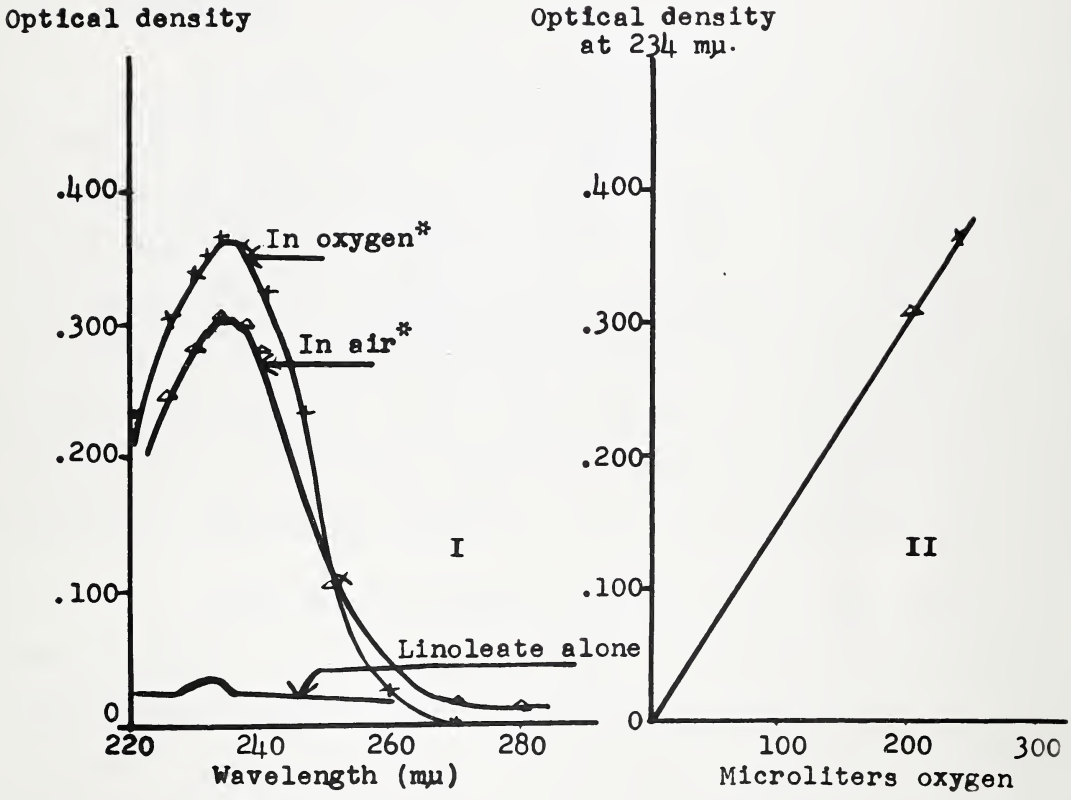


Fig. 12. I. Spectral absorption of the products of linoleate-lipoxidase oxidation.
II. Total dienes (OD_{234}) versus total oxygen uptake.

* The absorption of enzyme and substrate alone has been subtracted.

There was no evidence of carbonyl compounds as observed by Siddiqi and Tappel (49) at 279 mμ. Holman (27) concluded that carbonyl compounds might be decomposition products of linoleate hydroperoxides, and that they were formed especially at high temperatures (37° C.)

(f) Inhibitors

A study of enzymic inhibitors revealed that sunflower lipoxidase was not affected by 10^{-2} molar concentrations of azide or cyanide, nor by the same concentrations of ethylenediaminetetraacetate or diethyldithiocarbamate, indicating that the enzyme does not require a metal activator for its catalytic activity. Similarly, iodoacetamide (10^{-3} M) and p-chloromercuribenzoate (10^{-3} M) had no influence on enzyme activity. Consequently, there is no evidence for the participation of sulfhydryl groups, as reported for urd bean lipoxidase (50).

Antioxidants reduced the activity of the enzyme. Thus, catechol and α-naphthol at 10^{-2} M inhibited the reaction 28% and 100%, respectively. Contrary to expectation, 5×10^{-3} M α-tocopherol had no influence on the rate of oxygen uptake. This observation agrees with that of Siddiqi and Tappel (50), who found that urd bean and peanut lipoxidases were unaffected by 3×10^{-3} M α-tocopherol. Evidently, α-tocopherol was not a very efficient hydrogen donor for these oxidative systems. The presence of α-tocopherol resulted in

an increase in total oxygen uptake, suggesting that the compound was oxidized by the linoleate-sunflower-lipoxidase system. This could have been a non-specific oxidation by the peroxides.

The presence of potassium oleate (7.5×10^{-3} M) reduced the activity by 24%. Since potassium oleate alone was not oxidized, the inhibition of lipoxidase activity by this compound may be competitive, as shown by Mapson and Moustafa (39) for pea lipoxidase.

Ethanol (9.0%) depressed the rate by 53%, but had no influence on the total oxygen uptake. Ethanol could be expected to denature the enzyme protein and thus have a depressing effect on the rate of oxidation. However, it seemed to be a general property of sunflower lipoxidase that total oxidation was closely related to the apparent initial "active" enzyme concentration. Consequently, if ethanol had denatured some of the enzyme protein, the total uptake would have been less and not the same as in the absence of ethanol. Thus it would appear that the effect of ethanol may not have been due entirely to denaturation and inactivation of the enzyme.

(g) Copper-ion Activation

Cupric sulfate enhanced the rate and the total oxygen uptake during linoleate-lipoxidase oxidation (Table 4). Iron, manganese, magnesium and calcium were ineffective. A mixture of K linoleate, $CuSO_4$ (5×10^{-4} M), and bovine serum albumin showed no appreciable oxygen uptake. If bovine serum

albumin was replaced by the enzyme, $CuSO_4$ stimulated the rate by 20%, and the total uptake by 31%. Although cyanide had no effect on lipoxidase, it curtailed the effect of copper on the rate, and reduced considerably the total oxygen uptake (Table 4). The data also revealed an accentuated stimulation by copper after dialysis of E_P against cyanide.

Table 4. Copper-ion activation of linoleate-lipoxidase oxidation.

Reaction mixture*	% activation	
	Rate ($\mu l.\ O_2$/min.)	Total ($\mu l.\ O_2$)
Bovine serum albumin + $CuSO_4$ + K linoleate	No oxidation	
Enzyme + K linoleate	0	0
" + " + $CuSO_4$	20	31
" + " + " + Cyanide	0	17
After dialysis against cyanide:		
Enzyme + K linoleate + $CuSO_4$	45	38
" + " + $FeCl_3$	0	0

* Concentrations:
20 µmoles K linoleate; 1.0 µmole $CuSO_4$; 1.0 µmole $FeCl_3$; 20 µmoles NaCN. Phosphate buffer, pH 7.0. In oxygen.

The consistent increase in rate and total activity

in the presence of copper has not been observed with other lipoxidases. It is known, however, that copper greatly accelerates (57) the autoxidation of unsaturated fat (62) and soybean oil (13). Copper ions not only speed up the autoxidation, but also catalyze the decomposition of reaction products (29). Therefore, on the basis of the present experimental results, and the known catalytic effect of copper on autoxidation, it would appear that copper catalyzed a secondary reaction. Lipoxidase, or some other enzyme or protein present as an impurity, may have aided the catalytic effect of copper.

(h) Lipoxidase and Catalase Activity

When hydrogen peroxide was added to the reaction mixture, there was a rapid increase in pressure, an indication of oxygen evolution and a suggestion that catalase was present in the enzyme extract. Figure 13 shows that catalase activity had no effect on the initial rate of linoleate oxidation. Evidently, small amounts of hydrogen peroxide and slight catalase activity do not adversely affect the *in vitro* oxidation of linoleate by sunflower lipoxidase. Tappel (56) has shown that catalase and cytochrome c can catalyze oxidation of linoleate. However, since cyanide and azide did not influence the rate of linoleate oxidation, it would seem that catalase played a minor part.

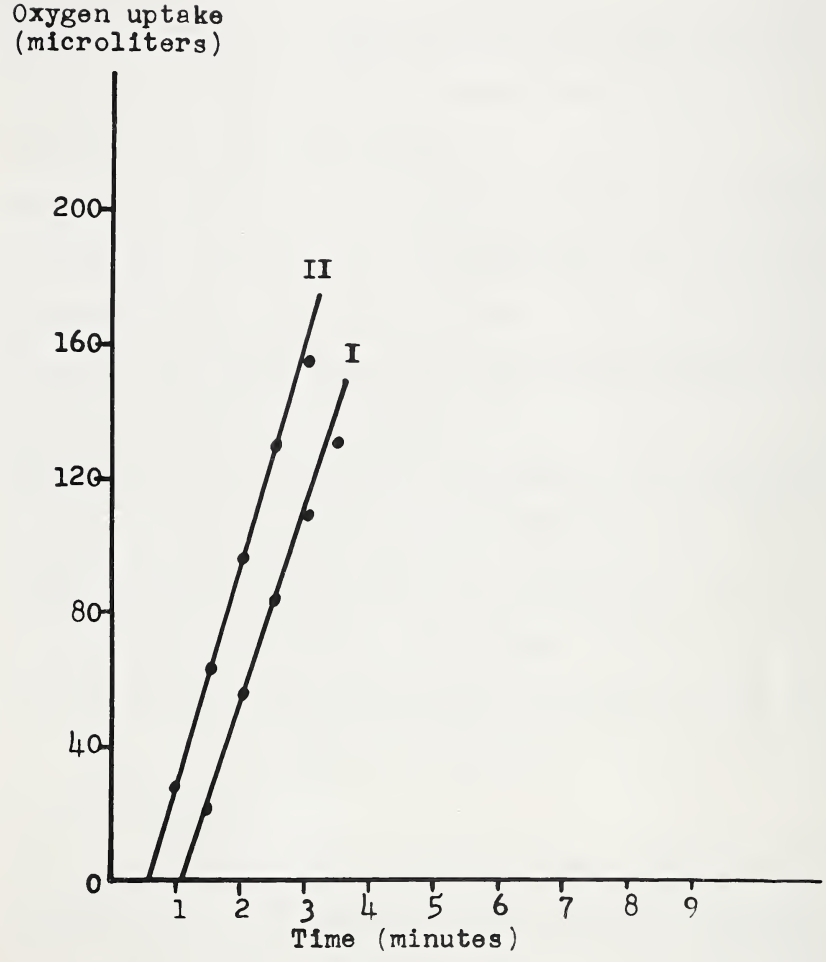


Fig. 13. Lipoxidase and catalase activity in sunflower seedling extracts.

Reaction mixture:
I. Enzyme (Ep) + 5 μmoles H_2O_2 + 20 μmoles K linoleate + phosphate buffer, pH 7.0
II. Enzyme (Ep) + 20 μmoles K linoleate + phosphate buffer, pH 7.0

SUMMARY

1. An active lipoxidase was obtained from sunflower seeds and seedlings.

2. Total oxidation of linoleate by sunflower lipoxidase was partly inhibited by ether-extracted oil from sunflower, flax and rape seeds.

3. During germination, lipoxidase activity of sunflower seedlings increased to a maximum and then decreased gradually with age of seedlings. The most rapid depletion of oil reserves in the seedlings coincided with maximum lipoxidase activity.

4. Sunflower lipoxidase was associated with a mitochondrial (15,000 x g), an intermediate (25,000 x g), and a microsomal fraction (100,000 x g), as well as the soluble cytoplasmic proteins. The enzyme activity could not be completely removed from the particulate fractions by washings, deoxycholate treatment, dialysis or acetone precipitation.

5. Kinetic studies were performed with a partly purified preparation of lipoxidase from sunflower seedlings.

6. The enzyme oxidized linoleate, but oleate was inert. The Km with linoleate as substrate was 1.64×10^{-3} M.

7. Maximum enzyme activity was obtained with 100% oxygen.

8. The optimum pH was 6.8.

9. Q_{10} was 1.25 and activation energy 3.5 kcal./mole.
10. The reaction products were conjugated dienes containing no measurable carbonyl compounds.
11. Lipoxidase activity was not affected by azide, cyanide, ethylenediaminetetraacetate, diethyldithiocarbamate, iodoacetamide, or p-chloromercuribenzoate.
12. Enzymic activity was inhibited by catechol, α-naphthol oleate, and ethanol, but α-tocopherol had no effect.
13. Copper sulfate stimulated the rate and total oxidation of the linoleate-lipoxidase system, but iron, manganese, magnesium and calcium were without effect.
14. Catalase did not appear to influence lipoxidase activity.

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